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(54) Title: SYNTHETIC GENE SEQUENCE FOR A SIGNAL PEPTIDE

(57) Abstract

The present invention relates to a process of transporting a desired protein into the endoplasmic reticulum of plant cells for transport within or outside the cell. This process utilizes a DNA molecule encoding for a hybrid protein. The encoded hybrid protein includes a first protein including a signal peptide derived from a putative vicilin signal peptide, its homolog, or a fragment thereof and a second protein which confers an extracellular protective effect or another desired effect in plant cells. This DNA molecule can be inserted into an expression system which can then be used to transform cells that will direct the desired second protein into its transport system.

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SYNTHETIC GENE SEQUENCE FOR A SIGNAL PEPTIDE

FIELD OF THE INVENTION

The present invention relates generally to recombinantly produced proteins in plant cells and a process of transporting such proteins into the endoplasmic reticulum of plant cells, where they can subsequently be transported outside the cell or deposited in specific intracellular compartments.

BACKGROUND OF THE INVENTION

In eucaryotic cells, the endoplasmic reticulum plays a number of important functions. The smooth endoplasmic reticulum, which lacks ribosomes, provides a site for synthesis and metabolism of fatty acids and phospholipids. The rough endoplasmic reticulum is studded with ribosomes which participate in the synthesis of cytoplasmic, membrane, and organelle proteins as well as in the synthesis of proteins to be secreted from the cell.

In producing a protein, which is to be secreted or deposited in a specific intracellular compartment, the gene containing the DNA encoding the desired protein is expressed. During such expression, RNA polymerase activates a control region on the gene which transcribes the DNA (i.e. encoded information) into messenger RNA. Transcription is ended by one or more "stop" codons on the gene. The messenger RNA is then translated into the encoded protein at the ribosomes bound to the rough endoplasmic reticulum. During translation of the information from the messenger RNA, a growing polypeptide emerges from the ribosome, passes through the membrane of the rough endoplasmic reticulum, and accumulates in its lumen or central cavity. Proteins destined for direct transport include a lead signal peptide section, which is what directs the protein to enter the endoplasmic reticulum, and is

then cleaved from the remainder of the protein after such entrance.

After synthesis, transported proteins in the rough endoplasmic reticulum move to the lumen cavity of the Golgi complex. In the Golgi complex, proteins and membrane constituents are directed to their appropriate destinations. This is achieved by enzymatically modifying the proteins with a "tag" (e.g., a carbohydrate or phosphate residue) to direct them to vesicles which transport the modified proteins outside the cell (or to intracellular compartments).

In plant cells, a variety of naturally occurring proteins are transported by the above-described mechanism. An example of such proteins are the *vicilins*, the major storage protein of developing legume seeds. *Vicilins* are synthesized as prepro-*vicilins* on membrane-bound ribosomes of the rough endoplasmic reticulum. As prepro-*vicilins* pass into the lumen of the rough endoplasmic reticulum, their signal peptides are removed, as recognized by T.J.V. Higgins and D. Spencer, "Precursor forms of Pea Gene Encoding Vicilin Subunits," 67 Plant Physiol 205-11 (1981), which is hereby incorporated by reference. The resulting peptides may then undergo various post-translational modifications. For example, in the lumen of the endoplasmic reticulum or in the Golgi complex, the protein may undergo glycosylation so that it is ready for intracellular transport, as recognized by B.S.L. Beevers, "Glycoprotein Metabolism in the Cotyledons of *Pisum Sativum* During Development and Germination," 57 Plant Physiol. 93-97 (1976) and R.A. Davey and W.F. Dudman, "The Carbohydrate of Storage Glycoproteins from Seeds of *Pisum Sativum*: Characterization and Distribution of Component Polypeptides," 6 Aust. J. Plant Physiol., 435-47 (1979), which are hereby incorporated by reference. After being directed into "protein bodies" within the cell, *vicilins* can undergo endoproteolytic cleavage to form a wide range of smaller molecular weight proteins, as disclosed by M.J. Chrispeels, T.S.V. Higgins, and D. Spence, "Assembly of Storage Protein Oligomers in the Endoplasmic Reticulum and Processing of the Polypeptides in

the Protein Bodies of Developing Pea Cotyledons," 93 J. Cell Biol. 306-13 (1982); R.R.D. Croy, J.A. Gatehouse, I.M. Evans, and D. Boulter, "Characterization of the Storage Protein Subunits Synthesized in vitro by Polyribosomes and RNA from Developing Pea (*Pisum sativum* L.), I." 148 Legumin. Planta 49-56 (1980); R.R.D. Croy, J.A. Gatehouse, I.M. Evans, and D. Boulter, "Characterization of the Storage Protein Subunits Synthesized *in vitro* by Polyribosomes and RNA from Developing Pea (*Pisum sativum* L.), II." 148 Vicilin Planta 57-63 (1980); J.A. Gatehouse, R.R.D. Croy, H. Morton, M. Tyler, and D. Boulter, "Characterization and Subunit Structures of the Gene Encoding *Vicilin* Storage Proteins of Peas (Pisum sativum L.)," 118 Eur. J. Biochem. 627-633 (1981); and D. Spencer and T.J.V. Higgins "The Biosynthesis of Legumin in the Maturing Pea Seeds," 1 Biochem. Int. 502-09 (1980), which are hereby incorporated by reference.

In recent years, advances in biochemistry have led to the preparation of recombinant cloning vehicles (e.g., plasmids) containing heterologous genes (i.e. DNA that encodes proteins not ordinarily produced by the host organism containing that vehicle). Briefly, a heterologous gene is first inserted into a closing vector, such as a plasmid. The vector is then transfected into the host cells, which are thereafter transformed. The tranformed cells then multiply to increase the population containing copies of the gene to be expressed in the host cell for production of a protein foreign to that cell.

In plant cells, recombinant DNA techniques have been utilized to produce a number of proteins suitable for extracellular secretion.

In J. Denecke et al., "Protein Secretion in Plant Cells Can Occur via a Default Pathway," 2 The Plant Cell, 51-59 (1990), the non-secretory enzymes phosphinothricin acetyl transferase, neomycin phosphotransferase II, and β-glucuronidase were secreted from plant cells when targeted to the endoplasmic reticulum lumen by signal peptide-mediated translocation. The signal peptides were derived from gene

fragments encoding for the pathogenesis-related protein 16(sPRI), a protein secreted in tobacco mosaic virus-infected tobacco leaves, and the prepro-peptide of cecropin B(sCEC), a peptide secreted into the hemolymph of the insect *Hyalophora cecropia*. It was found that the total level of enzyme expression was lower in secretion constructs (with the signal peptide sequence) than for unmodified genes which encoded for the enzyme alone.

In P. Lund et al., "Bacterial Chitinase is Modified and Secreted in Transgenic Tobacco," 91 Plant Physiol., 130-35 (1989), a gene encoding for bacterial chitinase and its natural signal sequence was cloned in plant cells. Bacterial chitinase was ultimately secreted from the plant cell.

G. Iturriaga et al., "Endoplasmic Reticulum Targeting and Glycosylation of Hybrid Proteins in Transgenic Tobacco," 1 The Plant Cell, 381-90 (1989) prepared a gene encoding for a hybrid protein of the putative signal peptide of patatin fused to β-glucuronidase. When the gene was cloned and expressed in plant cells, it was found that the patatin was cleaved from the resulting protein, and the β-glucuronidase was expressed. Again, however, the total level of β-glucuronidase was very low compared to non-secretory controls.

The use of genes encoding for a hybrid protein consisting of a signal peptide sequence fused to another protein for the purpose of secretion is thus well known in plant cells. However, these signal peptides are not satisfactory, because they do not achieve high expression levels and, in fact, decrease the expression level of the desired proteins. Accordingly, the need exists for effective signal peptide sequences which can maintain or enhance high expression levels of desired proteins in plant cells.

SUMMARY OF THE INVENTION

The present invention relates to a process of transporting a desired protein to the endoplasmic reticulum of

plant cells for secretion. This process utilizes a DNA molecule encoding for a hybrid protein which includes a first protein containing a signal peptide, as described below, which was derived from the putative published sequence for the vicilin signal peptide, or its homologs, or any fragments thereof fused to a second protein which confers an extracellular protective or other desired effect in plant cells. For example, the second protein may be a protein or fragment thereof which imparts bacterial, fungal, or freeze resistance outside the cell walls of plant cells transformed with an expression system in which that DNA molecule is cloned.

The DNA molecule encoding the hybrid protein of the present invention may be cloned into an expression vector (e.g., a plasmid) to form a recombinant DNA expression system. This system is then transfected into plant cells. The plant cells of the present invention are then transformed and grown in a suitable medium to increase the number of such transformed cells. The transformed cells thus express the DNA encoding the hybrid protein of the present invention. The hybrid protein includes the signal peptide described below, its homologs, or any fragment thereof which directs transport of the second protein into the endoplasmic reticulum. The signal peptide is cleaved off upon entry into the rough endoplasmic reticulum. The second protein, which is the protein of interest, is further modified in the endoplasmic reticulum lumen or in the Golgi complex and then secreted outside the plant cell or otherwise transported within the cell. Any plant cell transformed with this recombinant DNA expression system may then secrete the desired protein. This system can be used to secrete proteins which will condition resistance to bacteria, fungi, or frost, or will affect plant cell walls or function as markers or tags or color factors.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A shows the oligodeoxyribonucleotide signal sequence for the hybrid gene of the present invention.

Figure 1B shows the 5' end of the hybrid gene encoding for the signal peptide and β-glucuronidase, and the corresponding amino acids.

Figure 2A is a graph showing the β-glucuronidase activity for transformed leaf cells of transgenic tobacco plants.

Figure 2B is a graph showing the β-glucuronidase activity for transformed root cells of transgenic tobacco plants.

Figure 3A shows a chart of the distribution of transient β-glucuronidase activity in transformed tobacco protoplasts in the absence of tunicamycin.

Figure 3B shows a chart of the distribution of transient β-glucuronidase activity in transformed tobacco protoplasts in the presence of tunicamycin.

Figure 4 is a chart showing the total transient β-glucuronidase activity (pooled from all fractions) in transformed protoplasts in the presence of tunicamycin.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a process of transporting proteins to the endoplasmic reticulum of plant cells for secretion or other targeting within the cell. This process utilizes a DNA molecule encoding a hybrid protein. The hybrid protein includes a first part of the protein which contains a signal peptide derived from a putative sequence for the vicilin protein, its homologs, or any fragments thereof. This first protein is fused to a second protein which confers an extracellular protective effect in plant calls or any other desired effect such as for marking, color, cell wall modification, etc.

The signal peptide, its homologs, or fragments thereof is present to enable the hybrid protein to enter the cell's transport pathway via the endoplasmic reticulum. Upon

entry into the lumen, the signal peptide is cleaved from the second protein in the same fashion as the vicilin signal peptide would be separated from the vicilin protein to which it is fused in nature. The second protein can then be processed in the Golgi complex or in the endoplasmic reticulum lumen for extracellular secretion or transport within the cell.

Preferably, the DNA molecule of the present invention encodes for an amino acid signal sequence substantially corresponding to the sequence:

Met Leu Leu Ala Ile Ala Phe Leu Ala Ser Val Cys Val Ser Ser.

G.W. Lycett, A.J. Delauney, J.A. Gatehouse, J. Gilroy, R.R.D. Croy, and D. Boulter, "The Gene Encoding *Vicilin* Gene Family of Pea (*Pisum Sativum L.*): A Complete cDNA Coding Sequence for Preprogene Encoding *Vicilin*," 11 Nucleic Acids Res. 2367-80 (1983) predicts this putative signal peptide sequence from the cDNA sequence for a 47 kDA prepro-*vicilin* polypeptide, but does not disclose a specific gene encoding for this amino acid sequence. The accuracy of this amino acid sequence prediction is questionable. Moreover, the initial methione predicted by Lycett, et al. may, in fact, not be the true N-terminus of the peptide but may represent the truncation of the natural signal peptide. In any event, based on the amino acid sequence disclosed by Lycett, a synthetic DNA sequence encoding for that signal sequence has been prepared as follows:

ATG TTA TTA GCT ATT GCA TTT TTA GCA TCA GTT TGT GTT TCA TAC
ATT AAT CGA TAA CGT AAA AAT CGT AGT CAA ACA CAA AGT.

The second protein is a protein or fragment thereof which imparts a resistance to bacteria, fungi, or freezing or any other desired effect. The synthetic sequence derived from the *Pisum vicilin* signal peptide is effective in producing a hybrid protein which includes and carries a second protein

into the cell transport path. As a result the resistance provided by the second protein is imparted to plants extracellularly (i.e. outside the cell wall, particularly to extracellular surfaces of the plant cell walls).

Examples of proteins which may impart extracellular bacterial resistance to plants include lytic proteins. See, e.g., L. Destefano-Beltran, "Enhancing Bacterial and Fungal Disease Resistance in Plants: Application to Potato," Molecular Biology of the Potato, p. 205-21 (1990), which is hereby incorporated by reference.

Examples of proteins which may impart extracellular fungal resistance to plants include chitinases and glucanases. See, e.g., C. Castresana, "Tissue-Specific and Pathogen-Induced Regulation of a *Nicotiana plumbaginifolia* β-1,3-Glucanase Gene," 2 The Plant Cell, 1131-43 (1990); J. Neuhaus, "High-level Expression of a Tobacco Chitanase Gene in *Nicotiana sylvestris*. Susceptibility of Transgenic Plants to *Cercospora Nicotianae* Infection," 16 Plant Molec. Biol. 141-51 (1991); and L. Destefano-Beltran, "Enhancing Bacterial and Fungal Disease Resistance in Plants: Application to Potato," Molecular Biology of the Potato, p. 205-21 (1990), all of which are hereby incorporated by reference.

Examples of proteins which may impart extracellular antifreezing capability in plants include numerous fish antifreeze peptides. See, e.g., P.L. Davies, "Antifreeze Protein Genes of the Winter Flounder," 259 J. Biol. Chem. 9241-47 (1984), which is hereby incorporated by reference.

The second protein may include polygalacturonase, β-glucuronidase, other marker proteins, and their homologs, fragments, or portions thereof. Of these proteins, β-glucuronidase primarily serves as an example. The gene encoding for β-glucuronidase is a widely used marker gene having a well known sequence and known to be useful in forming N-terminal fusions. See, e.g., R.A. Jefferson, "Assaying Chimeric Genes in Plants: The GUS Gene Fusion System," 5 Plant Molecular Biology Reporter, 387-405 (1987) and R.A. Jefferson, "β-Glucuronidase from *Escherichia coli* as a

Gene-fusion Marker," 83 Proc. Nat. Acad. Sci., 8447-51 (1986), both of which are hereby incorporated by reference. The hybrid gene encoding for a hybrid protein containing β-glucuronidase and the amino acid which that gene encodes is shown in Figure 1B.

The polypeptide formed from the fusion of the first protein to the second protein is a hybrid in that the first and second proteins are not normally joined in nature. Likewise, the corresponding portions of the DNA molecule encoding each of these proteins makes the DNA molecule a hybrid.

The portion of the DNA molecule encoding the first protein is fused in proper orientation and correct reading frame to that part of the DNA molecule encoding the second protein. In Figure 1B, a linker sequence connects these portions of the DNA molecule.

The expression vector into which this fusion DNA molecule is inserted includes a promoter sequence which functions to regulate polypeptide expression. A wide variety of promoters may be utilized, such as the 35S promoter of cauliflower mosaic virus.

The DNA molecule encoding for the hybrid protein of the present invention is inserted into an expression system to which the DNA molecule is heterologous (i.e., not normally present). The heterologous DNA molecule is inserted into the expression system or vector in proper orientation and correct reading frame. Although a wide variety of expression systems can be utilized, the use of plasmids is particularly desirable. Suitable plasmids include *pBI426* and *pMON316*, preferably *pMON316*.

The expression system of the present invention can be used to transform virtually any crop plant cell under suitable conditions. Such cells are transformed with the DNA molecule of the present invention by conventional procedures. Cells transformed in accordance with the present invention can be grown *in vitro* in a suitable medium to produce the desired second protein. This protein can then be harvested or

recovered by conventional purification techniques. Alternatively, transformed cells can be regenerated into whole plants such that secreted proteins impart desired characteristics to the intact plants.

Regardless of whether the DNA molecule of the present invention is expressed in intact plants or in culture, expression of the desired protein follows essentially the same basic mechanism. Specifically, transcription of the DNA molecule is initiated by the binding of RNA polymerase to the DNA molecule's promoter. During transcription, movement of the RNA polymerase along the DNA molecule forms messenger RNA. As a result, the DNA molecule that encodes for the hybrid protein of the present invention is transcribed into the corresponding messenger RNA. This messenger RNA then moves to the ribosomes of the rough endoplasmic reticulum which, with transfer RNA, translates the messenger RNA into the hybrid protein of the present invention. During translation of the messenger RNA, the resulting hybrid protein passes into the lumen of the rough endoplasmic reticulum. After the signal sequence (i.e., the first protein, its homolog, or a fragment thereof) is cleaved at the site shown in Figure 1B, the second protein having the ability to confer desired effects will continue to advance into the lumen in an unfused state. This protein is then modified by glycosylation in the lumen or in Golgi complex. The modified protein is then secreted by the Golgi complex to a location outside the host cell or is transported within the cell to a specific site.

EXAMPLES

Example 1 -- Construction of Plant Expression Vector for Secretion of β-Glucuronidase

Sequences of component oligodeoxyribonucleotides (Figure 1A) coding for the N-terminus of the hybrid gene (DNA) encoding a signal peptide derived from the putative vicilin signal peptide and β-glucuronidase (Figure 1B) were prepared

by the following procedure. The oligodeoxyribonucleotides #1-#4 (Figure 1A) used to assemble the signal peptide sequence of *Pisum vicilin(s)* were synthesized using a conventional DNA synthesizer. The oligodeoxyribonucleotides #2 and #3 were phosphorylated by BRL T4 polynucleotide kinase. Complementary oligodeoxyribonucleotides of each segment were then annealed by combining 0.2 nmole of each, and incubating the mixture at 90°C for 10 minutes, at 65°C for 2 hours, and at room temperature (i.e. 32°C) overnight. The entire signal peptide sequence, with a 5' terminal *KpnI* site and a 3' terminal blunt end, was assembled from the two annealed segments (0.2 nmole each) using standard ligation procedures. The resulting DNA fragment was then purified from a polyacrylamide gel and used for construction of plant β-glucuronidase-secretion vector through a multiple-step procedure. The gene encoding β-glucuronidase was excised from the plasmid *pBI101.3* (Clontech Laboratories, Inc.) with *HindIII+SstI*, and ligated into the vector *pUC118*. See, J. Vieira and J. Messing, "Production of Single-stranded Plasmid DNA," 153 Methods Enzymol 3-11 (1987), which is hereby incorporated by reference, that had been cut with *HindIII+SstI*. In order properly to fuse the synthesized signal peptide-encoding nucleotide sequence to the N-terminus of the gene encoding β-glucuronidase, a *KpnI* linker (5'-GGGTACCC) and a *HindIII* linker (5'CCAAGCTTGG) were inserted into the unique *HindIII* and *SmaI* sites upstream of the gene encoding β-glucuronidase in the resulting *pUC118-β-glucuronidase* in the resulting *pUC118-β-glucuronidase* plasmid. The purified signal peptide fragment was then fused in-frame to the N-terminus of the gene encoding β-glucuronidase to form *pUC118-β-glucuronidase1*, by replacing the *KpnI-HindIII* multi-cloning fragment of the modified *pUc118-β-glucuronidase* with the assembled signal peptide-encoding fragment.

The fusion region of the hybrid gene in *pUC118-β-glucuronidase1* (Figure 1) was sequenced by the dideoxy chain-termination method of F. Sanger, S. Nicklen, and A.R. Coulson, "DNA Sequencing with Chain-terminating

Inhibitors," 74 Proc. Natl. Acad. Sci. USA 5463-67 (1977), the disclosure of which is hereby incorporated by reference, using the universal M13 primer. Finally, the *KpnI-EcoRI* fragment coding for the signal peptide/β-glucuronidase fusion was isolated from *pUc118-β-glucuronidase1* and cloned into the plant expression vector *pMON316*. See, S.G. Rogers, H.J. Klee, R.B. Horsch, and R.T. Fraley, "Improved Vectors for Plant Transformation: Expression Cassette Vectors and New Selectable Markers," 153 Methods Enzymol. 253-77 (1987), previously digested with *KpnI+EcoRI*. The resulting plasmid was designated *pMON316-β-glucuronidase1-1*. Plasmid *pMON316-β-glucuronidase1-2* was similarly constructed, according to the above-described procedures in an independent cloning experiment. The β-glucuronidase-encoding sequence, without the signal peptide, was closed into *pMON316* in both orientations to generate positive (*pMON316-β-glucuronidase(+)*) and negative (*pMON316-β-glucuronidase(-)*) controls for expression of the gene encoding for β-glucuronidase.

The fusion gene was then placed under the 35S promoter of cauliflower mosaic virus in a plant expression vector, and introduced into tobacco protoplasts for transient expression, and into tobacco leaf discs/NT1 cells for stable transformation, using conventional recombinant technology procedures.

Example 2 -- Analysis for the Presence of β-glucuronidase

Leaf discs from *in vitro* grown plantlets were transformed using an improved biolistic device of G.N. Ye, M. Daniell, and J.C. Sanford, "Optimization of Delivery of Foreign Gene into Higher Plant Chloroplasts," 15 Plant Mol. Biol. 809-19 (1990), which is hereby incorporated by reference, and transformants were selected on kanamycin medium following two days of incubation on non-selective medium. Transgenic plants from leaf discs were regenerated from shoots after transfer to a hormone-free tobacco medium. 20 μg tunicamycin per ml was added to the culture medium to inhibit

glycosylation of the fusion β-glucuronidase. Leaf or root extracts were prepared by grinding in the extraction buffer (50 mM Tris-HCl, pH 7.5/0.15 mg phenylmethylsulfonyl fluoride/0.3 mg dithiothreitol/0.3 mg bovine serum albumin, all per ml) and removing the cell debris by centrifugation. The β-glucuronidase fluorometric assay, described by J.H. Gould and R.M. Smith "A Non-destructive Assay for β-glucuronidase in the Media of Plant Tissue Cultures," 7 Plant Mol. Biol. Rep. 209-16 (1989), which disclosure is hereby incorporated by reference, was modified and used to measure enzymatic activity of β-glucuronidase in leaves (Figure 2A) or roots (Figure 2B) of transgenic plants. 2 µl samples were added to 50 µl of 4-methylumbelliferyl glucuronide (MUG) assay solution, and the mixtures were incubated at 37°C for 1 hour before stopping the reactions with 50 µl of stop buffer. 1 µl of each stopped reaction mixture was assayed in 1 ml of stop buffer using a DNA Fluorometer (Model TKO 100, Hoefer Scientific Instruments, San Francisco). β-glucuronidase activity was expressed as picomoles of 4-methylumbelliferone liberated per min per µg of total proteins. The results were from three independent experiments with each extract prepared from either leaves (Figure 2A) or roots (Figure 2B) of transgenic plants. In Figures 2A and 2B, GUS(-) and GUS(+) represent the plants transformed with *pMON316-β-glucuronidase*(-) and *pMON316-β-glucuronidase*(+), respectively, while GUS1-1 and GUS1-2 represent plants transformed with the similar vicilin(s) signal peptide/β-glucuronidase-encoding constructs *pMON316-β-glucuronidase1-1* and *pMON316-β-glucuronidase 1-2*.

Almost no β-glucuronidase activity was detected in leaves (Fig. 2A) or roots (Fig. 2B) of transgenic plants containing the fusion gene encoding for signal peptide/β-glucuronidase (*pMON316-β-glucuronidase1-1* or *pMON316-β-glucuronidase1-2*), whereas the normal level of β-glucuronidase activity was observed in the positive control plants transformed with *pMON316-β-glucuronidase*(+). In the same experiments, no β-glucuronidase activity was found in the

negative control plants transformed with the construct of an anti-sense β-glucuronidase gene [i.e. *pMON316-β-glucuronidase(-)*]. Since the fusion region of the hybrid gene encoding for β-glucuronidase was sequenced, and confirmed to be correct, the absence of β-glucuronidase activity in the plants containing the fusion gene must be attributed to the interference of gene expression by the addition of the signal peptide sequence. Alternatively, the gene fusion or post-translational modification of the fusion protein in the transport pathway could cause loss of β-glucuronidase activity.

Inactivation of the β-glucuronidase enzyme by the gene fusion was unlikely since β-glucuronidase had been shown to be remarkably tolerant to N-terminal modification. See R.A. Jefferson, "Assaying Chimeric Genes in Plants: the β-glucuronidase Gene Fusion System," 5 Plant Mol. Biol. Rep. 387-405 (1987); G. Iturriaga, R.A. Jefferson, and M.W. Bevan, "Endoplasmic Reticulum Targeting and Glycosylation of Hybrid Proteins in Transgenic Tobacco," 1 Plant Cell 381-90 (1989) ("Iturriaga"); and J. Denecke, J. Botterman, and R. Deblaere, "Protein Secretion in Plant Cells can Occur via a Default Pathway," 2 Plant Cell 51-59 (1990), which are hereby incorporated by reference. To test this possibility, experiments were carried out in which the transformants were treated with tunicamycin, an inhibitor of glycosylation. β-glucuronidase activity increased significantly, two days after tunicamycin treatment, in the tobacco containing the hybrid DNA molecule of the present invention, while the β-glucuronidase activity of the positive control plants expressing the β-glucuronidase encoding gene was not significantly affected by tunicamycin (Fig. 2B). Negative controls were unaffected by tunicamycin. This, together with the similar report in Iturriaga, provides strong evidence that the signal peptide efficiently directed β-glucuronidase to the rough endoplasmic reticulum, and that N-glycosylation occurred.

Example 3 - Secretion of β-glucuronidase into the Extracellular Space of Tobacco Cells

Protoplasts were prepared from leaves derived from seedlings of N. tabacum var Havana cv 423 grown in vitro at low light intensity, generally as described by J.I. Negy and P. Maliga, "Callus Induction and Plant Regeneration from Mesophyll Protoplasts of Nicotiana Sylvestris," 78 Z. Pflanzenphysiol 453-55 (1976), which disclosure is hereby incorporated by reference, with some modifications. Briefly, the sliced leaves were digested in an isolation medium containing 1.25% Cellulase R10 and 0.4% Macerozyme R10 (Karlan Chemical Corporation, Torrance, CA) at 26°C overnight. Debris was removed by filtering the mixture through a 59 μm mesh and the protoplasts were then collected at the surface of the isolation medium after a spin at 100xg for 5 minutes. The protoplasts were resuspended in the isolation medium, and 1 ml of W5 solution, as described by I. Negrutiu, R. Shillito, I. Potrykus, G. Biasini, and F. Sala, "Hybrid Genes in the Analysis of Transformation Conditions," 8 Plant Mol. Biol. 363-73 (1987) ("Negrutiu"), which is hereby incorporated by reference, was layered on top of the protoplast suspension. After centrifugation at 100xg for 5 minutes, the protoplasts at the interface were transferred to new tubes and washed with W5 solution. The isolated protoplasts were transformed with the plasmid constructs described above using the polyethylene glycol method of Negrutiu. The transformed protoplasts were then cultured at the final density of $1x10^6$ protoplasts/ml in the culture medium (containing 20 μg tunicamycin/ml where indicated) at 26°C in the dark. The transformed tobacco protoplasts were fractionated into extracellular, cytoplasmic and membrane-associated fractions about 50 hours after the polyethylene glycol-mediated transformation. The extracellular fraction (the culture medium) was collected as supernatant from the protoplast cultures following centrifugation at 100xg for 5 minutes. The pelleted protoplasts were washed once in W5 solution and lysed by

osmotic shock in an appropriate volume of extraction buffer (50 mM Tris-HCl pH 7.5/0.15 mg phenylmethylsulfonyl fluoride/0.3 mg dithiothreitol/0.3 mg bovine serum albumin, all per ml). The cytoplasmic fraction was recovered as supernatant following centrifugation at 16,000xg at 4°C for 5 minutes. The pellet was washed once in extraction buffer, and resuspended and sonicated in the same buffer with 0.1% Triton X-100. The membrane-associated fraction was then obtained as supernatant after removal of residual cell debris.

β-glucuronidase fluorometric assay was conducted to measure enzymatic activity of β-glucuronidase in different fractions of transformed protoplasts. The results of this assay are plotted in Figures 3A and 3B where Ex, C, and M refer to the extracellular, cytoplasmic, and membrane-associated fractions, respectively.

In the absence of tunicamycin, no β-glucuronidase activity was detected in either extracellular or membrane-associated fractions of the protoplasts expressing the gene encoding for β-glucuronidase or the hybrid thereof (Fig. 3A). However, after tunicamycin treatment, a significant amount of β-glucuronidase activity was observed in the extracellular and membrane fractions of the protoplasts transformed with the hybrid gene but not those transformed with the gene encoding β-glucuronidase (Fig. 3B). These results suggest that some of the hybrid β-glucuronidase enzyme was directed to the rough endoplasmic reticulum, glycosylated in the lumen of the rough endoplasmic reticulum, and secreted from the protoplasts. Secretion of β-glucuronidase enzyme fused to the signal peptide of *Pisum vicilin(s)* (which normally directs protein deposition into protein bodies) supports previous studies showing that secretion can occur via a "default pathway," as disclosed by R. Bassuner, A. Huth, R. Manteuffel, and T.A. Rapoport, "Secretion of Plant Storage Globulin Polypeptides by *Xenopus Laevis Oocytes*," 133 Eur. J. Biochem 321-26 (1983); M.M. Bustos, V.A. Luckow, L.R. Griffing, M.D. Summers, and T.C. Hall, "Expression, Glycosylation, and Secretion and Phaseolin in a Baculovirus System," 10 Plant Mol. Biol. 475-88 (1988); J. Denecke, J. Botterman, and R. Deblaere, "Protein Secretion in Plant Cells can Occur via a

Default Pathway," 2 Plant Cell 51-59 (1990) ("Denecke"), which are hereby incorporated by reference. That is, proteins migrate nonspecifically from the lumen of the rough endoplasmic reticulum via the Golgi complex to outer cell surfaces unless they contain signals for directed transport to other cell compartments. Since only a small proportion (~20%) of β-glucuronidase enzyme produced in the transformed protoplasts was transported into the extracellular medium, most of the rough endoplasmic reticulum-processed β-glucuronidase enzyme may have been transported intracellularly to be deposited in membrane-bound vesicles. This is supported by the observed increase in cytoplasmic β-glucuronidase activity of the protoplasts producing the hybrid β-glucuronidase after tunicamycin treatment (Fig. 3). Alternatively, inefficient secretion may reflect a structural restriction of the β-glucuronidase enzyme, limiting its transport via the secretory pathway, as described by Denecke.

In contrast to the results from transgenic tobacco plants (Fig. 2), a comparable amount of β-glucuronidase activity was found in the cytoplasmic fractions of tobacco protoplasts transiently expressing either the gene encoding for β-glucuronidase or the hybrid β-glucuronidase thereof whether or not tunicamycin was present (Fig. 3). Similar results were obtained with most transgenic *Nicotiana tabacum* cells when stained with the β-glucuronidase substrate mixture. The apparent difference between levels of β-glucuronidase activity in leaf or root cytoplasm versus callus or protoplast cytoplasm can best be explained by the very high levels of expression of the gene encoding for β-glucuronidase found in the latter two cell types. At very high expression levels, the transport system appears to become saturated or partially blocked, resulting in accumulation of β-glucuronidase in the cytoplasm. For example, the protoplasts transiently expressing the hybrid gene produced 10-30 fold more β-glucuronidase protein (Fig. 4) than the transgenic plants containing the same construct (Fig. 2).

Example 4 - Enhanced expression of the hybrid gene encoding for signal peptide/β-glucuronidase by the signal peptide

We have also observed that total β-glucuronidase activity in the hybrid gene transformants is substantially greater than the positive control gene encoding β-glucuronidase transformants after treatment with tunicamycin (Fig. 4). This was determined by transforming $2x10^6$ protoplasts with 40 μg of DNA of respective constructs as described in Example 3. This data was inconsistent with that reported by S.J. Rothstein, C.M. Lazarus, W.E. Smith, D.C. Baulcombe, A.A. Gatenby, "Secretion of a Wheat Alpha-amylase Expressed in Yeast," 308 Nature 663-65 (1984) and Denecke, which showed that their signal peptides dramatically reduced synthesis of hybrid proteins. These different findings may result form the use of different signal peptides, which could affect the synthesis of the attached proteins negatively or positively. It is conceivable that the nucleotide sequence and codon usage at the N-terminus of a protein have a strong influence on transcription and translation efficiency, respectively. This data indicates that the signal peptide used in this study allows higher levels of expression of passenger proteins -- a significant improvement over prior art efforts utilizing a signal peptide sequence to transport an attached heterologous protein into the endoplasmic reticulum for secretion.

Although the invention has been described in detail for the purpose of illustration, it is understood that such detail is solely for that purpose, and variations can be made therein by those skilled in the art without departing from the spirit and scope of the invention which is defined by the following claims.

WHAT IS CLAIMED:

1. A DNA molecule encoding for a hybrid protein comprising a first protein comprised of a signal peptide sequence derived from a vicilin signal peptide, its homologs, or a fragment thereof fused to a second protein which confers an extracellular protective effect or another desired effect to plant cells.

2. A DNA molecule according to claim 1, wherein said second protein is selected from the group consisting of proteins, their homologs, or fragments thereof which impart bacterial, fungal, insect, and freeze resistance to extracellular surfaces of plant cells, which modify cell walls, or are otherwise useful.

3. A DNA molecule according to claim 2, wherein said second protein is selected from the group consisting of chitanases, glucanases, protease inhibitors, insect-specific neurotoxins, antifreeze peptides, lytic peptides, polygalacturonidase, and fragments or portions thereof.

4. A DNA molecule according to claim 2, wherein said second protein is β-glucuronidase, a homolog, fragment, or portion thereof, or a similar marker or color protein.

5. A DNA molecule according to claim 1, wherein said DNA molecule encodes for an amino acid signal sequence substantially comprising the sequence:

Met Leu Leu Ala Ile Ala Phe Leu Ala Ser Val Cys Val Ser Ser.

6. A DNA molecule according to claim 5, wherein said DNA molecule has a sequence substantially corresponding to the nucleotide sequence:

ATG TTA TTA GCT ATT GCA TTT TTA GCA TCA GTT TGT GTT TCA TCA TAC AAT AAT CGA TAA CGT AAA AAT CGT AGT CAA ACA CAA AGT AGT.

7. A recombinant DNA expression system comprising an expression vector into which is inserted a heterologous DNA

molecule encoding for a hybrid protein comprising a first protein comprised of a signal peptide derived from a vicilin signal peptide, its homolog, or a fragment thereof fused to a second protein which confers an extracellular protective effect or another desired effect to plant cells.

8. A recombinant DNA expression system according to claim 7, wherein said heterologous DNA is inserted into said vector in proper orientation and correct reading frame.

9. A recombinant DNA expression system according to claim 7, wherein said second protein is selected from the group consisting of proteins, their homologs, or fragments thereof which impart bacterial, fungal, insect, and freeze resistance to extracellular surfaces of plant cells, which modify cell walls, or are otherwise useful.

10. A recombinant DNA expression system according to claim 7, wherein said second protein is β-glucuronidase, a homolog, or a fragment or portion thereof, or a similar marker or color protein.

11. A recombinant DNA expression system comprising the DNA molecule of claim 5.

12. A recombinant DNA expression system comprising the DNA molecule of claim 6.

13. A cell transformed with the recombinant DNA expression vector of claim 7.

14. A cell transformed with a recombinant DNA expression vector containing the DNA molecule according to claim 5.

15. A cell transformed with a recombinant DNA expression vector containing the DNA molecule according to claim 6.

16. A hybrid protein comprising a first protein comprised of a signal peptide derived from a vicilin signal peptide, its homolog, or a fragment thereof fused to a second protein which confers an extracellular protective effect or another desired effect in plant cells.

17. A hybrid protein according to claim 16, wherein said second protein is selected from the group consisting of

proteins, or fragments thereof, which impart bacterial, fungal, insect, and freeze resistance to extracellular surfaces of plant cells, which modify cell walls, or are otherwise useful.

18. A hybrid protein according to claim 17, wherein said second protein is β-glucuronidase, a homolog or a fragment thereof, or a similar marker or color protein.

19. A hybrid protein according to claim 16, wherein said first protein is encoded by a gene encoding for an amino acid signal sequence substantially comprising the sequence:

Met Leu Leu Ala Ile Ala Phe Leu Ala Ser Val Cys Val Ser Ser.

20. A process of producing a recombinant hybrid protein comprising:

growing host cells transformed with a recombinant DNA expression vector comprising an expression system into which is inserted a heterologous DNA molecule encoding a hybrid protein comprising a signal peptide derived from a vicilin signal sequence, its homolog, or a fragment thereof fused to a second protein having an extracellular protective effect or another desired effect in plant cells and

expressing the heterologous DNA molecule in said host cells to produce the hybrid protein.

21. A process according to claim 20 further comprising:

harvesting the hybrid protein produced by said expressing.

22. A process according to claim 20 further comprising:

passing the hybrid protein produced by said expressing into the endoplasmic reticulum of the host cell, wherein the signal peptide is cleaved from the hybrid protein during said passing;

conveying the second protein having an extracellular protective effect or any other desired effect in

plants to the Golgi complex of the host cell, wherein the second protein is modified by glycosylation; and

secreting the modified second protein from the Golgi complex to a location extracellular to the host cell or otherwise transporting the second protein within the cell.

23. A process according to claim 20, wherein said DNA molecule encodes for an amino acid signal sequence substantially comprising the sequence:

Met Leu Leu Ala Ile Ala Phe Leu Ala Ser Val Cys Val Ser Ser.

24. A process according to claim 23, wherein said DNA molecule has a sequence substantially corresponding to the nucleotide sequence:

ATG TTA TTA GCT ATT GCA TTT TTA GCA TCA GTT TGT GTT TCA TCA TAC AAT AAT CGA TAA CGT AAA AAT CGT AGT CAA ACA CAA AGT AGT.

25. A cellular transport process comprising:

directing a protein into the endoplasmic reticulum of a plant cell with a signal peptide derived from a vicilin signal peptide, its homolog, or a fragment hereof.

26. A hybrid fusion gene encoding for a signal peptide sequence derived from a vicilin signal peptide fused to another protein.

27. A hybrid fusion protein comprising a signal peptide sequence derived from a vicilin signal peptide fused to another protein.

SEGMENT A

Oligo*1

5'-CATGTTATTAGCTATTGCA

Oligo*2

CATGGTACAATAATCGATAACGTAAAAAAA-5'

SEGMENT B

Oligo*3

5'TTTTTTAGCATCAGTTTGTTTCATC

Oligo*4

TCGTAGTCAAACACAAAGTAG-5'

FIG. 1A

KpnI

5'-C ATG TTA TTA GCT ATT GCA TTT TTA GCA TCA GTT

CATGG TAC AAT AAT CGA TAA CGT AAA AAT CGT AGT CAA

Met Leu Leu Ala Ile Ala Phe Leu Ala Ser Val

LINKER SEQUENCE

TGT GTT TCA TCA GCT TGG GGG TAC GGT CAG TCC CTT

ACA CAA AGT AGT CGA ACC CCC ATG CCA GTC AGG GAA

Cys Val Ser Ser Ala Trp Gly Tyr Gly Gln Ser Leu

CLEAVAGE SITE

ATG TTA

TAC AAT

Met Leu

DNA ENCODING FOR β-GLUCURONIDASE

β-GLUCURONIDASE

FIG. 1B

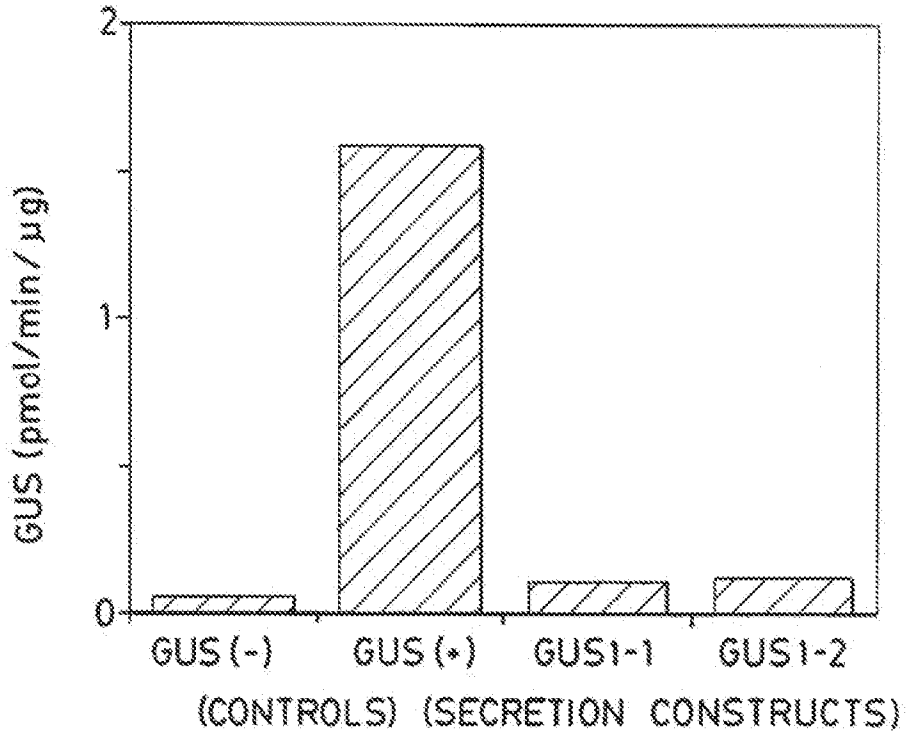

2
1
0
GUS (pmol/min/μg)
GUS(-)
GUS(+)
GUS1-1
GUS1-2
(CONTROLS) (SECRETION CONSTRUCTS)

FIG. 2A

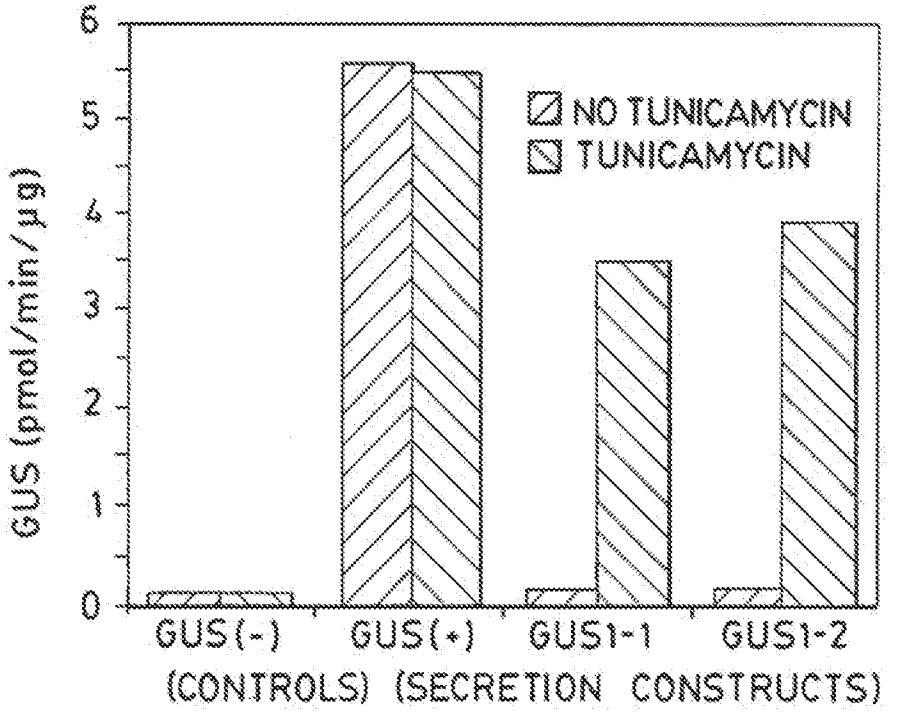

6
5
4
3
2
1
0
GUS (pmol/min/μg)
NO TUNICAMYCIN
TUNICAMYCIN
GUS(-)
GUS(+)
GUS1-1
GUS1-2
(CONTROLS) (SECRETION CONSTRUCTS)

FIG. 2B

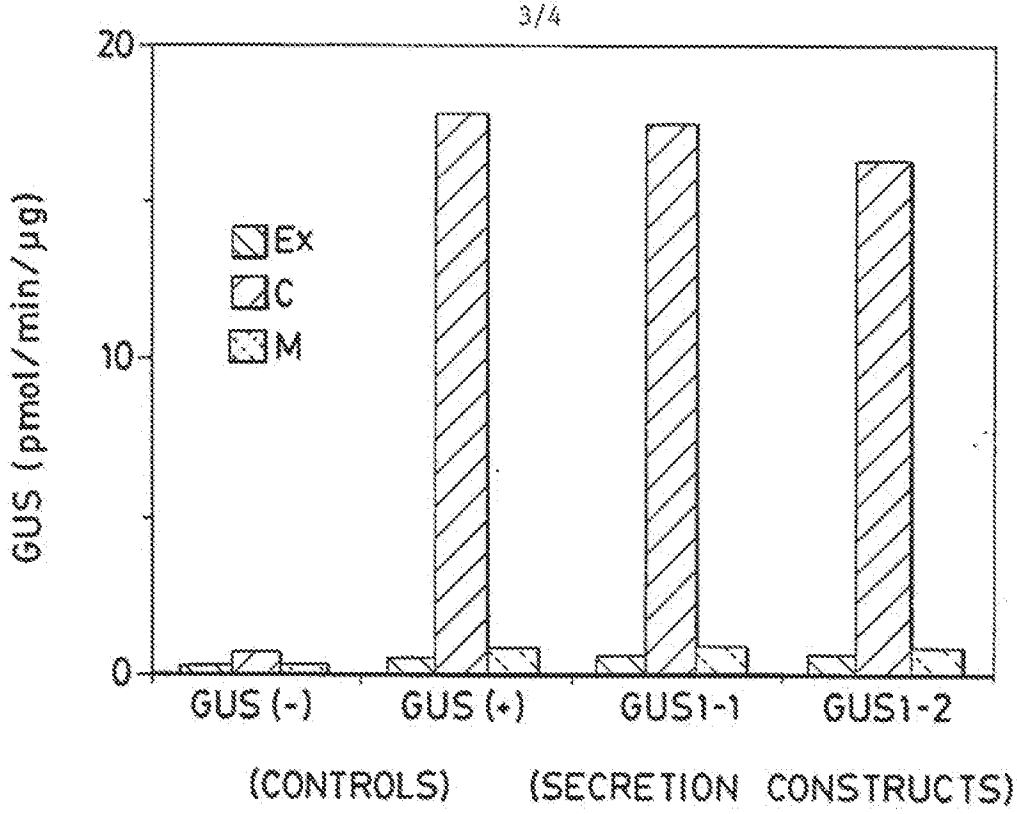
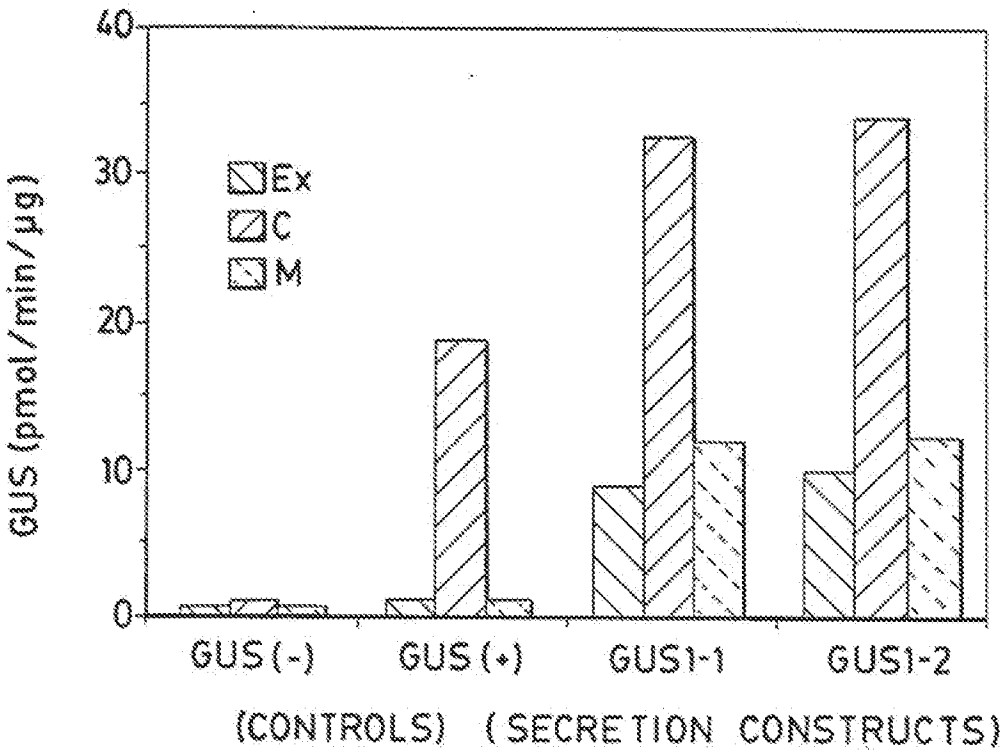

20
10
0
GUS (pmol/min/µg)
Ex
C
M
GUS (-)
GUS (+)
GUS1-1
GUS1-2
(CONTROLS)
(SECRETION CONSTRUCTS)

FIG. 3A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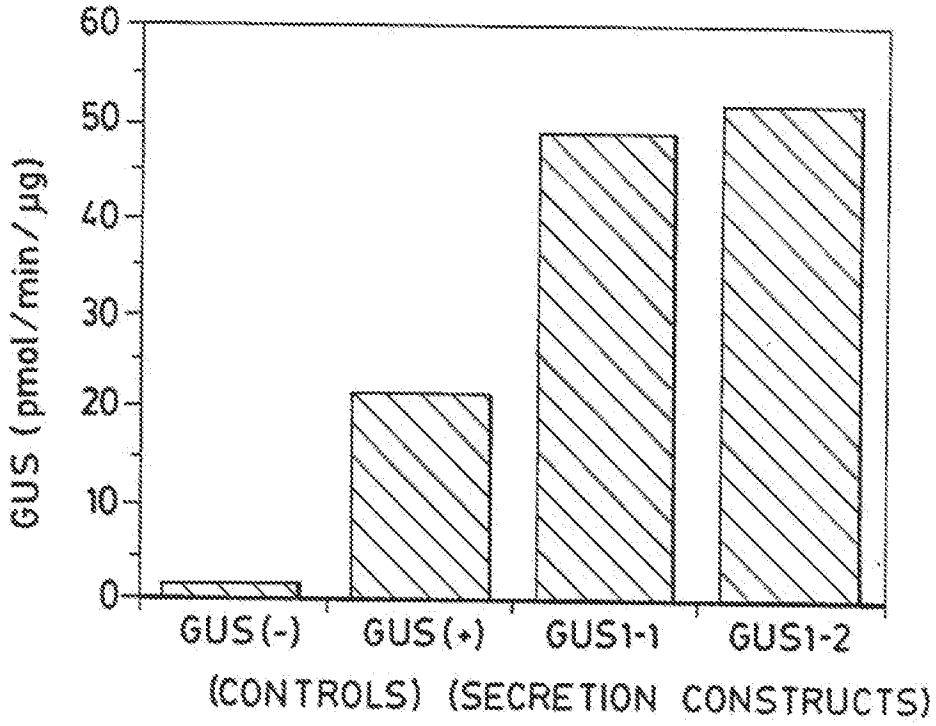
30
20
10
0
GUS (pmol/min/µg)
Ex
C
M
GUS (-)
GUS (+)
GUS1-1
GUS1-2
(CONTROLS)
(SECRETION CONSTRUCTS)

FIG. 3B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50
40
30
20
10
0
GUS (pmol/min/μg)
GUS(-)
GUS(+)
GUS1-1
GUS1-2
(CONTROLS)
(SECRETION CONSTRUCTS)

FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US93/01494

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12P 21/06, 21/02; C12N 5/00, 15/00; C07H 17/00
US CL :435/69.1, 69.7, 69.8, 70.1, 240.4, 320.1; 536/23.4, 23.2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 69.7, 69.8, 70.1, 240.4, 320.1; 536/23.4, 23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG
search terms: vicilin, signal peptide, signal sequence, endoplasmic reticulum, transport, protein processing

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 4,940,840 (SUSLOW ET AL) 10 JULY 1990, see entire document.	1-21, 23-24
X	PLANT MOLECULAR BIOLOGY, Volume 11, issued 1988, T. J. V. Higgins et al, "The sequence of a pea vicilin gene and its expression in transgenic tobacco plants", pages 683-695, see entire document.	25

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
30 March 1993	27 APR 1993

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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US93/01494

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	THE PLANT CELL, Volume 1, issued March 1989, G. Iturriaga et al, "Endoplasmic reticulum targeting and glycosylation of hybrid proteins in transgenic tobacco", pages 381-390, see entire document.	1-27
Y	GENE, Volume 99, No. 1, issued 01 March 1991, M. De Loose et al, "The extensin signal peptide allows secretion of a heterologous protein from protoplasts", pages 95-100, see entire document.	1-27
Y	NUCLEIC ACIDS RESEARCH, Volume 11, No. 8, issued 1983, "The vicilin gene family of pea (Pisum sativum L.): a complete cDNA coding sequence for preprovicilin", pages 2367-2380, see entire document.	1-27
Y	PLANT PHYSIOLOGY, Volume 91, issued 1989, P. Lund et al, "Bacterial chitinase is modified and secreted in transgenic tobacco", pages 130-135, see entire document.	3